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Renin processing and secretion in adrenal and retina of transgenic (mREN-2)27 rats

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Renin processing and secretion in adrenal and retina of transgenic (mREN-2)27 rats. Two extrarenal tissue sources of renin were studied using quantitative assays and immunocytochemical methods during 12 hours following binephrectomy (BNx) in anesthetized hypertensive homozygous Ren-2 transgenic (TG) rats maintained off hypotensive drugs for three weeks. Compared to normal rats, circulating active renin was depressed 50% in conscious TG rats and prorenin was 5- to 10-fold higher. Post-BNx, arterial active and prorenin increased progressively to 10-fold, at which time adrenal venous outputs were 0.1 and 20 mGU/min, respectively. The ratio of active to prorenin (3.1 ± 0.6%) remained unchanged with increasing plasma levels. Thus, either intrinsic enzyme activity of the transgenic prorenin contributed a constant proportion to the measured active renin, or processing to mature renin was coupled to prorenin synthesis and secretion in extrarenal tissues. In the TG rat eye, renin protein labeling was localized throughout retinal Müller cells with prosequence more obvious posteriorly, consistent with directional processing. Immunogold studies are in progress. In adrenal following BNx, labeling for renin and prosequence increased uniformaly in all zones of the cortex and in scattered medullary chromaffin cells. In cortex, both renin and prosequence were strongly located in intramitochondrial dense bodies. In chromaffin cells, renin labeling was present in both cytoplasmic vesicles and electron-dense granules, while prosequence was predominantly in cytoplasmic vesicles, consistent with processing of prorenin prior to storage in chromaffin granules.

The major sites of extrarenal renin expression and possible secretion in humans are adrenal gland and reproductive tract [1]. We have also recently localized renin to the retinal macroglia Müller cell and presented morphological evidence consistent with secretion into retinal capillaries [2]. But to date secretion of extrarenal renin into blood *in vivo* has been convincingly shown for only ovary, uterus and salivary gland in selected species [3, 4].

Compared with kidneys the renin content of adrenal glands and eyes is low (ratio of 10^5:6:1 in the rat) [2] requiring immunocytochemical and arterio-venous secretion studies to be conducted at their detection limits. However, the transgenic (TG) REN-2 rat which displays severe hypertension associated with amplified extrarenal expression of a mouse renin transgene (*Ren-2*) together with abundant secretion of its proform [5, 6] presents an opportunity to explore more precisely the physiology of tissue renin synthesis, processing and secretion.

In order to study extrarenal renin production, binephrectomy (BNx) is usually performed to eliminate any renal renin but this procedure enhances adrenal gland renin content in both TG and normal rats [7, 8] although to date actual secretion has not been demonstrated. Importantly, following adrenalectomy (ADRx) in BNx TG rats, continuing high levels of both active and prorenin persist in the circulation [9] indicating other secretory sources.

In the present work we localize renin and prorenin immunocytochemically in adrenal and retina of TG rats and quantitate the adrenal secretion of each during 12 hours following BNx. These experiments form part of an objective to characterize the processing and secretion of renin from all tissues of TG and normal rats to further elucidate the biology of tissue renin. Whether the REN-2 TG hypertensive rat represents an amplified but otherwise qualitatively valid model of the normal state is a contentious issue addressed in this continuing work.

Methods

Two hundred to 250 g female homozygous REN-2 TG rats (Max-Delbruck-Centrum, Berlin-Buch, Germany) were studied three weeks after removing maintenance angiotensin converting enzyme inhibitor (ACEI, lisinopril 10 mg/ml) from the drinking water. Systolic blood pressure (tail cuff) of conscious animals was measured before withdrawing ACEI and again three weeks later just prior to experimentation. A tail vein blood sample (400 μl into 5 U of heparin) was collected at this time. Blood was handled as described below.

Operative procedures and experimental protocols

Rats were anesthetized with pentobarbitone (Nembutal 6 mg/100 g body wt i.p.) prior to surgical procedures and maintained deeply sedated thereafter. The trachea and carotid artery were cannulated and the kidneys removed rapidly through flank incisions. The flank incisions were closed and arterial renin levels were followed from 30 minutes to 12 hours post-BNx. At seven to eight hours the left flank was reopened and the left genital vein was ligated and the cut end of the left renal vein was cannulated with PE 50 tubing. The animal was then heparinized (80 U/100 g i.v. initially and 20 U 2 hourly), the renal vein was ligated at its junction with the vena cava and the adrenal venous blood flowing from the cannula was recirculated into the jugular vein via a short loop. This preparation has been deployed for the collection of adrenal venous blood [10] and allows the measurement of blood flow by air bubble transit time.

A-V plasma collections and renin assay

Carotid arterial and adrenal venous blood (each 200 μl) were collected simultaneously from the heparinized animal at intervals

to 12 hours following BNx. Blood was replaced by volume with isotonic saline. An aliquot (100 μl) of each blood sample was immediately added to a cocktail of protease inhibitors on ice (EDTA, N-ethyl maleimide, benzamidine) [11] and both aliquots were then centrifuged cold. The plasmas were separated and stored at −30°C prior to assay of active and total renin. The proteases inhibitors were added to prevent spontaneous activation of prorenin and these aliquots were used for active renin assay. Total renin was assayed after trypsin activation of prorenin using aliquots without inhibitors. The optimum concentration, time and temperature conditions for trypsin activation were established to be 2 mg/ml for 10 minutes at 4°C. Enzymatic renin activity was then assayed after incubation (30 min, 37°C, pH 7.4) by RIA of generated angiotensin using 24 hours BNx rat plasma as the substrate in the presence of further protease inhibitors and expressed in Goldblatt units (GU) relative to the international hog renin standard as previously described [11]. Prorenin was derived as total minus active. Angiotensinogen (aogen) was measured after incubation of plasma with unpurified mouse salivary gland renin. Conditions for maximum generation and survival of angiotensin I were established as previously stated [11].

Statistical testing

One-way ANOVA followed by a Newman-Keuls test and paired *t*-tests were applied and justified as indicated in the text.

Tissue fixation

Adrenal glands from three female, 12-hour post-BNx TG rats (see above) were immersed in Bouin's fluid for three hours for immunohistochemistry. Five male transgenic REN-2 rats (200 to 250 g) that had received ACEI since weaning were anesthetized with Nembutal (6 mg/100 g body wt i.p.) and prepared for vascular perfusion as previously described [12]. The abdominal aorta was cannulated and vascular perfusion was commenced at 200 mm Hg using either Bouin's fixative (for immunohistochemistry, $N = 3$) or 4% paraformaldehdye with 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 (for immunogold, $N = 2$), and the inferior vena cava adjacent to the renal vein was severed. After five minutes of vascular perfusion (250 ml of fixative), adrenal glands, eyes and kidneys were removed and post-fixed for two hours in the same fixative. For immunohistochemistry, tissues were dehydrated, routinely processed and embedded in paraffin. For immunogold, tissues were rapidly dehydrated in graded alcohols and embedded in London White resin (Bio-Rad, Cambridge, Massachusetts, USA). One micrometer sections were stained with methylene blue, the block was further trimmed and ultrathin sections (70 nm) were cut with diamond knives and placed on 0.5% Formar-coated nickel grids (EF Fullam, Inc., Schenetady, New York, USA).

Immunohistochemistry

Three micrometer sections of tissues were incubated for 20 minutes with normal goat serum (NGS) diluted 1:10 with 0.1 M phosphate buffered saline (PBS) at pH 7.4. The sections were incubated for 18 hours at 4°C with either 1:20 to 1:200 polyclonal anti-mouse renin protein antiserum [12], 1:20 to 1:200 polyclonal anti-rat renin fusion protein antiserum [13], 1:20 to 1:400 polyclonal mouse prosequence antiserum [12] or 1:10 NGS. All antisera were diluted in PBS. Antibody binding was visualized by an avidin-biotin peroxidase technique using diaminobenzidine as the chromagen. The sections were stained briefly in Mayer's hematoxylin and differentiated in Scott's tap water, dehydrated, cleared and mounted.

Immunogold

Methods for immunogold have been previously described [12]. Briefly, ultrathin sections of adrenal gland cortex and medulla were incubated for 10 minutes with 1:10 NGS. The sections were then incubated for 18 hours at 4°C with either 1:10 to 1:20 polyclonal anti-mouse renin antiserum, 1:10 polyclonal anti-rat renin antiserum, 1:20 to 1:50 polyclonal anti-mouse prosequence antiserum or 1:10 NGS. All antisera were diluted in PBS. After thorough rinsing in PBS (3 × 20 min), sections were incubated with goat anti-rabbit IgG-coated particles (15 nm, Biocell, Cardiff, UK) diluted 1:25 with PBS for one hour at room temperature. Sections were rinsed with PBS (3 × 20 min) and distilled water (2 × 5 min). The sections were stained with uranyl acetate (5 min) and lead citrate (5 min) and examined in a Phillips CM12 electron microscope.

Antisera specificities

The renin protein and prosequence antisera specifically label juxtaglomerular cells of the kidney, the adrenal cortex, Müller cells of the eye and granulated convoluted tubules of the mouse submandibular gland [2, 12, 13]. The mouse prosequence antiserum is raised against a synthetic oligopeptide epitope in the prosequence [12] and reacts only with the proform. The mouse and rat renin protein antisera display cross reactivity.

Results

Conscious blood pressure of TG rats

Mean tail artery systolic pressure of 200 to 250 g TG female rats raised from weaning on ACEI in drinking water was 118 ± 3.2 mm Hg ($N = 8$). Three weeks off ACEI, mean pressure was 141 ± 5.0 mm Hg ($P < 0.001$, $N = 7$, unpaired t = test).

Effect of BNx on arterial renin in TG rats

Immediately following BNx the plasma levels were not different from conscious pre-operation tail vein levels indicating that neither anesthesia nor BNx caused acute release of renin. Within 30 minutes of BNx, active renin and prorenin were significantly elevated in arterial plasma and increased progressively to reach 10-fold control at 12 hours post-BNx (Fig. 1). Despite the progressive increase in both renin forms the concentration ratio did not change from the conscious value of 3.1 ± 0.6. Figure 1 also shows that following BNx, aogen increased progressively in a manner similar to normal rats [14] despite the fact that active renin increased 10-fold.

Renin release from the adrenal gland of TG rats

Figure 1 shows that at 8, 10 and 12 hours post-BNx, the three times at which it was measured, a positive veno-arterial (V-A) difference for both active renin and prorenin was present across the left adrenal gland. The left adrenal venous outflow rate averaged 130.4 ± 4.8 μl/min ($N = 6$) at 8 to 12 hours post-BNx and calculated secretion rates from two adrenals were 0.1 and 20 mGU/min for active and prorenin, respectively, at 12 hours. Aogen did not display a V-A difference.

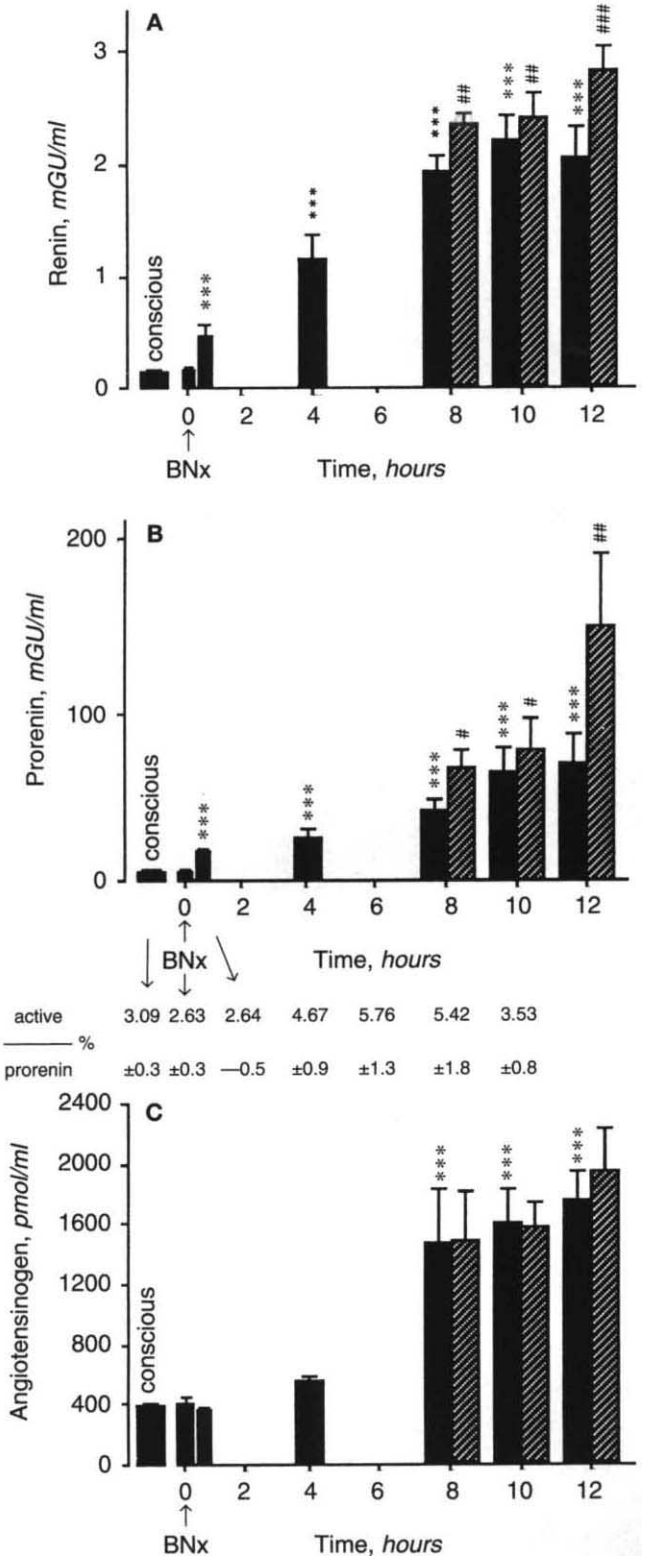

Fig. 1. *Arterial (black) and venous (hatched) plasma active renin (A), prorenin (B) and angiotensinogen (C) before (conscious) and to 12 hours following BNx in 5 female Ren-2 TG rats.* Values are plotted as mean ± SEM. The ratios of active to prorenin are shown with respect to time. The increase in arterial values (*** $P < 0.001$) was tested by one-way ANOVA compared with the zero time post-BNx value. The veno-arterial difference was tested by paired *t*-test (# $P < 0.05$).

Immunocytochemistry

In the adrenal glands of TG rats, intense and specific labeling for anti-rat renin protein (1:100), anti-mouse renin protein (1:100, Fig. 2A) was seen in cells of the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) and in some medullary cells. In the cortex, labeling appeared granular and was strongest around nuclei, while in medullary cells labeling was evenly distributed throughout the cytoplasm. A similar pattern of labeling was seen for anti-mouse prorenin (1:200), but labeling was more intense (Fig. 2B). Following 12 hours of BNx in TG rats labeling for anti-mouse (1:200) and anti-rat (1:200) renin protein and anti-mouse prorenin (1:400) was more intense in the adrenal cortex and the scattered medullary cells (Fig. 2 C, D). In the retina of TG rats, specific labeling for anti-mouse renin protein (1:200) was seen only in macroglial Müller cells (not shown). Labeling was more intense than in Müller cells of Sprague-Dawley rat eyes, particularly in the endfeet which made numerous contacts with retinal blood vessels. Intense labeling for anti-mouse prorenin (1:200) was observed between photoreceptor cell nuclei, corresponding to Müller cell processes.

Immunogold

In TG rat adrenal cortex, cortical cells contained numerous mitochondria (Fig. 2E), with intense labeling for anti-mouse (1:20) and anti-rat (1:20) renin protein and anti-mouse prorenin (1:20) in intramitochondrial dense bodies of mitochondria in ZG, ZF and ZR (Fig. 2F). Some labeling was seen over the remaining mitochondrial matrix. In the adrenal medulla, some cells contained chromaffin granules (Fig. 2G). In these cells the anti-rat (1:20) and anti-mouse (1:20) renin protein antisera specifically labeled cytoplasmic vesicles and occasional small electron-dense granules distributed throughout the cytoplasm (Fig. 2H). Labeling for anti-mouse prorenin (1:20) was mainly detected in cytoplasmic vesicles (Fig. 2I).

Discussion

Increases in circulating active and prorenin in 24 hour BNx TG Ren-2 rats have been reported recently together with evidence that tissue sources in addition to adrenal are involved, particularly for active renin [7, 8]. The present experiments show the time course of the increases and allow the conclusion that by 12 hour post-BNx, the two adrenals are secreting active renin at rates equivalent to the kidneys of normal pentobarbital anesthetized rats. Thus at 12 hours post-BNx the arterial level of active renin (2 mGU/ml) is about sixfold higher than for normal Sprague-Dawley rats (0.32 mGU/ml) [11], and for prorenin 200-fold higher [15]. The rate of adrenal renin secretion (0.1 mGU/min) is comparable to that found for normal rat kidneys (deduced from a clearance rate of 2.9 ml plasma/kg/min) [16]. It must be noted, however, that the assayed active renin is probably wholly transgenic mouse renin and further, that this *in vitro* activity could be due exclusively to the prorenin content if it possesses 3 to 4% of its potential enzyme activity, as has been reported for recombinant human renin [17]. The fact that the ratio of active to prorenin in arterial plasma remained statistically unchanged at 2.6 to 5.7% despite 10-fold increases in each, indicates either that Ren-2 mouse prorenin possesses a constant low enzymatic activity in plasma, or that processing to mature active Ren-2 renin and its subsequent

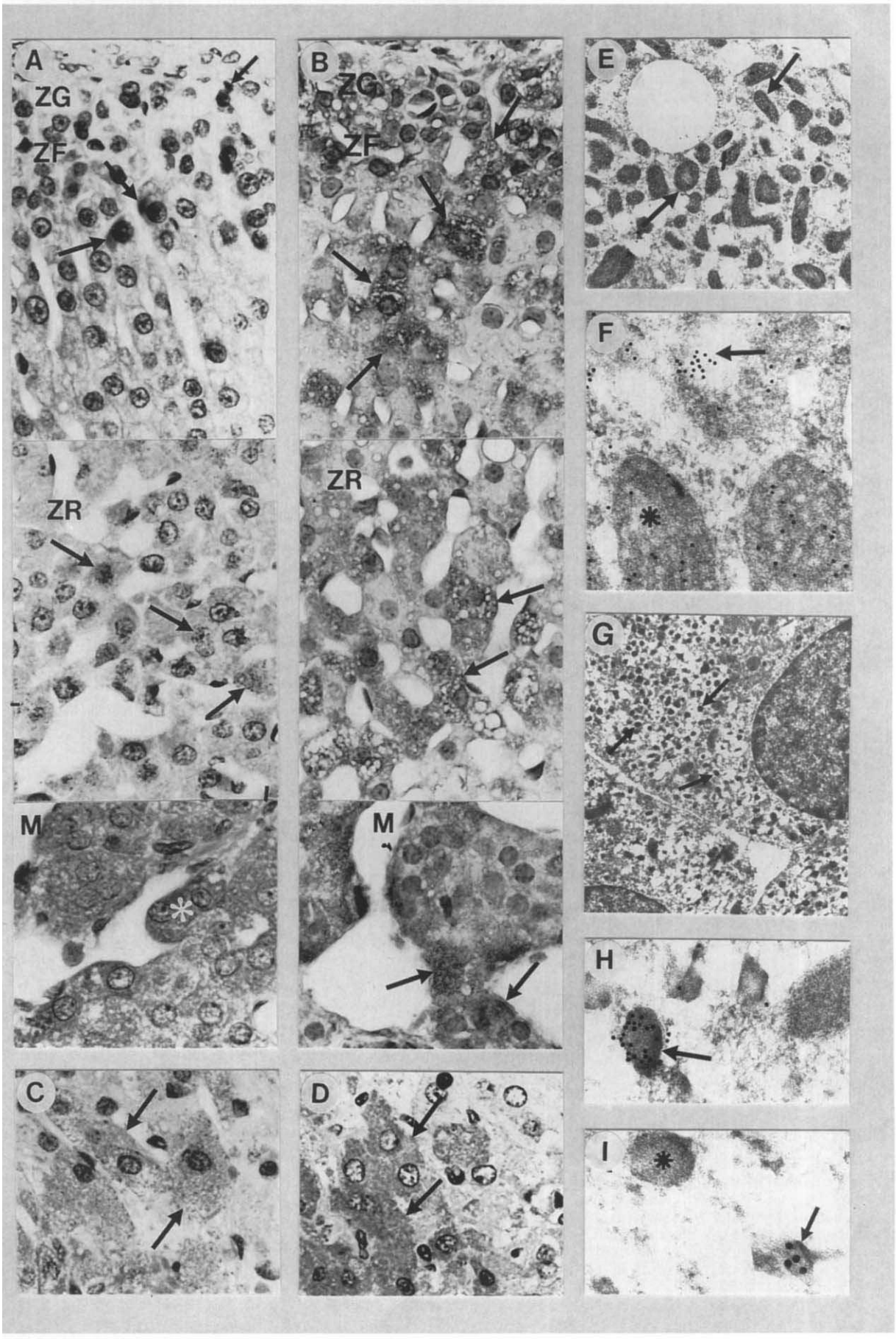
A
ZG
ZF
ZR
M
C
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Fig. 2. *Three micrometer paraffin sections of adrenal glands from TG rats labeled with antisera to renin protein and prorenin.* Mayer's hematoxylin counterstain. Abbreviations are: ZG, zona glomerulosa; ZF, zona fasiculata; ZR, zona reticularis, M, medulla. (Magnification ×264). **A.** Labeling for anti-mouse renin protein (1:100, arrows) is intense and granular in perinuclear regions of ZG, ZF and ZR cells. Labeling is seen in some medullary cells (arrow) and is evenly distributed throughout the cytoplasm. **B.** Labeling for anti-mouse prorenin (1:200, arrows) in ZG, ZF, ZR and some medullary cells is more intense than for renin protein. **C.** Adrenal medulla following 12 hour BNx. Labeling for anti-mouse renin protein (1:200, arrows) appears more intense and granular than in controls. Labeling is seen throughout the cell cytoplasm. **D.** Adrenal medulla following 12 hour BNx. Labeling for anti-mouse prorenin (1:200, arrows) appears more intense than in controls. Transmission electron micrographs of transgenic rat adrenal gland. Uranyl acetate and lead citrate counterstain. **E.** Zona reticularis. Numerous mitochondria (arrows) are distributed throughout the cell cytoplasm. (Magnification ×10,000). **F.** Higher magnification of zona reticularis cell shown in Fig. 2E. Labeling for anti-mouse renin protein (1:20) is intense in intramitochondrial dense bodies (arrow). Some labeling is also seen over the mitochondrial matrix (asterisk). (Magnification ×70,000). **G.** Medullary chromaffin cells. Numerous small electron-dense granules (arrows) are found throughout the cytoplasm. (Magnification ×7600). **H.** Higher magnification of a medullary chromaffin cell shown in Fig. 2G. Occasional electron-dense granules are labeled for anti-mouse renin protein (1:20, arrow). (Magnification ×56,000). **I.** Higher magnification of a medullary chromaffin cell shown in Fig. 2G. Cytoplasmic vesicles labeled for prorenin (1:20, arrow) are found in the cell cytoplasm. Most large electron-dense granules are not labeled for prorenin (asterisk). (Magnification ×123,000).

secretion is tightly coupled to synthesis and secretion of prorenin in the tissues of origin.

In the normal rat, mouse and human eye, renin but not prosequence labeling can be seen in macroglial Müller cells, which span almost the entire width of the retina and are closely associated with retinal blood vessels at their anterior surface [2]. In TG rats, prorenin labeling was clearly seen and was confined to the posterior part of the Müller cell, while labeling for renin extended throughout the cell cytoplasm, consistent with renin being processed in the direction of retinal blood vessels. Immunogold studies are in progress to determine the precise processing and secretory pathways in these cells. In the adrenal the localization of renin and prorenin labeling to intramitochondrial dense bodies in all cortical adrenal zones is consistent with recent publications [18, 19], and together with the presence of angiotensin type 1 receptors on the membranes of mitochondria [19] indicate a possible role for a renin-angiotensin system as an intacrine system in steriodogenesis. In the normal and TG rat, labeling for renin and prorenin were also found in some cells of the adrenal medulla. Immunogold studies revealed that the active renin is located in chromaffin granules, while prorenin is confined to cytoplasmic vesicles. The processing enzymes involved in the activation of renin within medullary cells, verification of the cell type, and the contributions of chromaffin granules and cortical mitochondrial renin to post-BNx plasma levels and the normal functions of adrenal renin all remain to be determined.

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